National Birth Defects Prevention Study
Protocol for the Collection of Biological Specimens

This protocol serves as a guide for the collection of biological specimens as part of the National Birth Defects Prevention Study, and has been agreed upon by the Biologics Subcommittee of the Centers for Birth Defects Research and Prevention.

I. Specimen Collection Methods

All Centers will use the dry buccal cytobrush method of specimen collection. Use of this method will be assessed on a regular basis to ensure that this is the optimal technology for our study.

II. Preparation and Shipping of Cheek Cell Sample Kits to Study Participants

Collaborating Centers will prepare and send cheek cell sample kits to all mothers following completion of the computer-assisted telephone interview (CATI) except those who refuse to participate in the collection of biological specimens. At the end of the CATI, interviewers read a script describing the cheek cell collection and mothers are offered the opportunity to review the kit before deciding if they want to participate in the collection of cheek cells. A cheek cell sample kit is sent to the mother that includes a cheek cell sample kit introduction letter, informed consent forms, cheek cell collection instructions, a money order, labeled colored envelopes containing labeled cytobrushes, and a self-addressed, stamped, bubble-lined mailing envelope. BioKit is used to generate labels for each cytobrush and to track shipping and receiving of the cytobrushes.

Each Center follows their own protocol for preparing kits to be shipped. It is suggested that a minimum of two staff members from each Center check the kits to verify that all components are included using the correct interview language and that the study ID on the brush labels correspond with the name of the mother on the introduction letter and on the outside mailing label. A checklist or label should be prominently affixed to either the consent form or return mailing envelope to remind mothers to return consent forms with the brushes. The last five digits of the nine-digit study ID should be added to the consent form as an additional quality control. When the consent form is returned, the partial study ID should be compared to the name on the consent form to verify that the kit was sent to the intended family. If the kit was not sent to the intended family, the mix-up will be resolved by asking the family to collect cells a second time.

Each Center follows their own standard protocol for placing reminder phone calls and sending reminder letters for cheek cell sample kits. An example of such a protocol from the Atlanta Center follows: A cheek cell sample kit is sent to the mother a day or two after the telephone interview. If the completed kit is not returned to the Center after 14 days, reminder call #1 is made. Approximately four attempts are made to reach the mother by phone during a one week time period. If none of the phone attempts result in contact with the mother, a reminder letter that includes an address correction form with stamped return envelope is sent to the family. If the kit has not been received 14 days after either phone contact with the mother or after sending the reminder letter, reminder call #2 is made. Approximately four attempts are made to reach the mother over a one week period. If none of the phone attempts result in contact with the mother, a decision letter is sent that includes an address correction form with stamped return envelope and informs the mother that no further attempts will be made to contact her if she does not return the form or call the number provided. If the kit is not returned and no response from the mother is received 14 days after the decision letter is sent, the family status will be changed to interview only in the clinical database. The cytobrushes that are not returned to the Center should be expired in BioKit. If the mother requests another kit at any time during this process, a new kit is generated with unique IDs using the Resend Kit option in the Admin portion of BioKit and it is sent from the initiating Center. Any prior kits generated for the family that have not been returned should be expired in BioKit. If a kit is returned after it has been marked as expired, it can be un-expired using the Alter data/Alter study participant option in the Admin portion of BioKit. Following shipment of the first kit, all subsequent kits that are sent to the mother for the initial collection are referred to as “resends”. ***There is no limit on the number of resends allowed but there is a maximum of three money orders sent to one family.***

Center's protocols: buccal cell reminder letters and phone calls

III. Specimen Log in and Shipping to the Central Lab using BioKit

It is important to minimize the amount of time that buccal cell specimens are at ambient temperature. Multiple freeze thaw cycles of the buccal cell specimens should also be avoided. Therefore, we recommend using freezers that are not "frost-free" and are able to maintain a temperature of at least –40^{o}C. Both the local cytobrushes and the CDC Central Lab brushes should be logged into the Center's BioKit database upon arrival. Local cytobrushes and the CDC Central Lab brushes should be segregated by family into separate Ziploc bags as outlined below and stored frozen. Central Lab brushes should be batch shipped to the CDC on dry ice monthly.

1. When a kit is received and opened at your Center, you should find up to 3 different colored envelopes and consent forms inside the larger mailing envelope. The colored envelopes should each contain two cytobrushes. In BioKit, go to Reception, Check-In and select the kit number received. Click on Load Kit and the brush numbers will appear on the right. The default "Date Received" will be the date you are entering the kit into BioKit. If you are entering the kit on a date other than when it was received, change the Date Received to the correct date. For each colored envelope, if there is a date on the envelope, enter it into the Date Collected box for that family member. Open the colored envelope and remove one cytobrush. Either check the appropriate box next to the brush number or scan the brush label into BioKit. Remove the second cytobrush and check or scan it into BioKit. As you check in each brush, it will automatically be designated as either Central Lab or local but you will not be able to view the designations until after all brushes are checked-in. If there is only one brush, it will be designated Central Lab. Repeat this process for the remaining colored envelopes. The consent form box must also be checked for each family member if a signed consent was received. If you know a particular brush will not be collected, you can check the Expire Now box next to that brush number. After all brushes have been checked in, click on the Confirm Reception of Kit button. Pop-up windows will appear to complete the process. One of the pop-up windows will list which brushes are local and which are Central Lab brushes. Return the brushes designated as Central Lab to their respective colored envelopes. Transfer the brushes designated local to a suitable container, agreed upon by your lab.

2. Place all colored envelopes from one family into a Ziploc bag. In order to reduce the amount of handling when you prepare your shipment to the Central Lab, it is recommended that you record the individual brush ID numbers of the Central Lab cytobrushes on the outside of the colored envelopes or the Ziploc bag using black permanent marker. This will allow you to quickly "see" what's inside without actually opening all the colored envelopes.

3. Repeat this exact process for all brushes received and store frozen at a temperature of -40°C or colder in a NON frost-free freezer. It will be useful to store the CDC Central Lab brushes in a dedicated container in the freezer from one shipment date to the next.

4. To reiterate, you are checking in both the CDC Central Lab brush and the local brush from one family into the database. As part of the check-in process, the computer will automatically assign which brushes are for the CDC Central Lab and which brushes are for local use. The CDC Central Lab brushes are physically separated from the local brushes, with the Central Lab brush remaining in the original colored envelope and the local brush being transferred to a new container. All colored envelopes from one family are placed in a large Ziploc bag with brush IDs from one family written on the outside of the Ziploc bag in permanent marker.

5. On your Center's designated shipment date (see schedule below), go to the Shipment Generation screen in BioKit and select shipment to the Central Lab. BioKit will generate a shipping manifest based on the brushes that have been logged in since your last shipment to the Central Lab. Print this list so you can include it with your shipment.

6. Keeping the Ziploc bags containing CDC Central Lab-designated cytobrushes in the freezer or on dry ice, and using the BioKit-generated shipping manifest, ***inspect that the IDs on the Ziploc bags match the IDs on the manifest.*** Proceed until all brushes on the manifest have been accounted for. Keep one family per Ziploc bag.

7. Transfer all Ziploc bags with CDC Central Lab brushes to an approved shipping container with a ***minimum of 10 pounds*** of dry ice (more during warm weather). An example of an approved shipping container would be a waterproof, shock absorbing, temperature insulating carton of a size sufficient to comfortably fit all the brushes plus the dry ice. You can purchase shipping containers in various sizes and shapes. Fisher Scientific and such vendors have many to choose from. Even though you may re-use these types of shipping containers multiple times, it is not appropriate to use or reuse an ordinary cardboard box for shipping purposes. Please remember, in order to accommodate all your brushes and the required amount of dry ice, you may ship more than one box to the Central Lab. Each Center must arrange and pay for their own shipping using cooperative agreement funds.
   Shipping container should be labeled as follows:

   Attn: Cindy Sturchio
   Phone: 770-488-4313
   Centers for Disease Control and Prevention-Centralized Lab
   4770 Buford Highway, MS F-24
   Bldg 110, Room 3204
   Atlanta, Georgia 30341

   A label specifying "Dry Ice for Medical Purposes; 5 lbs", and "Call Help Desk before Rejecting (**UPS** Hazardous Materials: 1-800-554-9964 Ext. 4932)" needs to be affixed to the container as well. Pack a ***minimum of 10 lbs*** dry ice in the shipping container even though the label states "5 lbs" for OSHA regulation compliance. If the shipping box has been used before, obliterate all pre-existing markings. Inside the shipping container, include a copy of the shipping manifest in a Ziploc bag for protection.

8. Ship to CDC on **Monday ONLY** (or Tuesday, if Monday is a holiday) once a month. Each Center has been assigned a ***shipping schedule*** as follows:
   Week 1 (first Monday of the month): Texas and Arkansas
   Week 2 (second Monday of the month): California, Utah, and New York
   Week 3 (third Monday of the month): Iowa and North Carolina
   Week 4 (fourth Monday of the month): CDC and Massachusetts

9. Send an e-mail notification to Cindy Sturchio when the shipment is made. Include an electronic copy of the shipping manifest.
   E-mail address: arp4@cdc.gov
   Subject line: Center code "buccal shipment to arrive"
   Example: GA17 buccal shipment to arrive
   If there are fewer than 10 brushes, you may hold them and ship with the next month's shipment to save shipping costs. If no brushes are shipped, send an e-mail with the subject line: Center code "no shipment this month"

IV. Central Lab Specimen Receiving using BioLab

Once your shipment arrives at the Central Lab, the shipping manifest will be scanned in and used to identify all brushes in the shipment. All brushes will be scanned into the Central lab database (BioLab) and verified against the manifest. A "Specimens Received" report will be generated depicting the EXACT contents received and e-mailed to the appropriate personnel. When confirmation is received at the originating Center, use BioKit and go into Shipments, Confirmation and confirm the shipment was received by the Central lab. Any discrepancies between what was thought to have been shipped and what was actually received will be highlighted. It will be each Center's responsibility to resolve these issues of their own accord.

The Central Lab will retain your shipping container for one week to allow sufficient time for the dry ice to dissipate and have any and all shipping discrepancies resolved. The Central Lab will then return your shipping container to you via US Mail after obliterating dry ice labels. New shipping containers will become necessary as their condition deteriorates.

V. Central Lab DNA Extraction

All data related to DNA extraction and quality control (DNA concentration, allele sizes, family relationships etc.) will be stored in the Central Lab database (BioLab). Specimens will be extracted using a manual phenol chloroform-based method that yields DNA of high quality and purity. DNA from all samples will be aliquotted into two tubes containing 50ul each (large volume tubes). Some samples will undergo further subaliquotting as specified in the quality control section.

SOP: Buccal Cell Extraction with Phenol Chloroform

VI. Quantitative PCR (RNAse-P)

All specimens will be quantified using RNAseP real time TaqMan PCR and the Applied Biosystems' 7500 Real-Time PCR System. Using this method, we are able to determine the initial copy number of a target template by analyzing the cycle-to-cycle changes in fluorescence signal as a result of template amplification during PCR.

SOP: Real Time Quantitative PCR Assay: RNAseP

VII. Quality Control

Microsatellite makers have been agreed upon by the Biologics subcommittee: D13S317 and D7S1797 -- further information available through the Genome Database -- **http://gdbwww.gdb.org**

SOP: Qiagen Multiplex Master Mix Microsatellite PCR Assay

SOP: NBDPS Fragment Analysis Genotyping: ABI 3100

Decisions regarding the criteria for acceptance of a specimen into the DNA repository have been finalized by the Biologics Committee.

All samples will be quantified using RNAseP real time TaqMan PCR. A cut-off value and indeterminate gray zone have been established. Samples with DNA concentrations below the cutoff [1ng/ul] will be flagged. We recommend that these samples be recollected. Each family will be asked for no more than one recollect, using a ***Center-approved standard letter***. Families should receive another money order for $20 when additional samples are requested. If there is no hard refusal, a maximum of three cheek cell sample kits should be sent to recollect the family's DNA with a money order maximum of three. When no sample is recollected, the original "indeterminate" sample may be used. Any "failed" samples will be stored in the hope that future technological advances make them usable.

Samples will be amplified using primer sets for two markers, D13S317 and D7S1797, in a multiplex PCR. Samples that pass quantitative PCR and microsatellite PCR will be included in analysis of family relationships using microsatellite allele sizes.

Specimen Scoring Algorithm

	Q-PCR* Pass (>1.0 ng/ul)	**Q-PCR Indeterminate (between 0.1 and 1.0 ng/ul)**	**Q-PCR Fail (<0.1 ng/ul)**
	Proceed to family	**Recollect** Note that DNA yield may be	**Recollect** Note that DNA yield may

Both STR pass	analysis, aliquot unless inconsistent Q-PCR =PASS D7S=PASS D13S=PASS **Specimen=Pass**	too low to yield reliable results?? Q-PCR =Indeterm D7S=PASS D13S=PASS **Specimen=Indeterm**	be too low to yield reliable results?? Q-PCR=FAIL D7S=PASS D13S=PASS **Specimen=Indeterm**
Only one STR passes	**Recollect** Note that there may be a problem with the DNA? Q-PCR =PASS D7S=PASS or FAIL D13S=PASS or FAIL **Specimen=Indeter**	**Recollect** Note that DNA yield may be too low to yield reliable results?? Q-PCR =Indeterm D7S=PASS or FAIL D13S=PASS or FAIL **Specimen=Indeterm**	**Recollect** Note that DNA yield may be too low to yield reliable results?? Q-PCR=FAIL D7S=PASS or Fail D13S=PASS or Fail **Specimen=Indeterm**
Both STRs fail	**Recollect** do not aliquot, undetermined DNA quality problem Q-PCR =PASS D7S=FAIL D13S=FAIL **Specimen=Fail**	**Recollect** do not aliquot Q-PCR =Indeterm D7S=FAIL D13S=FAIL **Specimen=Fail**	**Recollect** do not aliquot Q-PCR=FAIL D7S=Fail D13S=Fail **Specimen=Fail**

Specimens are scored based on a combination of Q-PCR and microsatellite results and are given an overall DNA quality and quantity score of pass, indeterminate, or fail

1. Fail
   a. Specimen is negative for both markers.
      i. Sample passed QPCR: Make two large volume aliquots; do not subaliquot. Repeat amplification one time; if still unsuccessful for both primer sets, the sample will be submitted to CASPIR as two large volume aliquots. The samples will be given a new collection name and stored separately within the NBDPS project in the CASPIR database. The family will be asked to submit additional samples, using a Center-approved standard letter. Each family will be asked for no more than one recollect following the standards stated previously. The information regarding problems with amplification will be noted in the Central lab database (BioLab) since this sample has an undetermined DNA quality problem.
      ii. Sample was considered indeterminate or failed QPCR: Take the same actions as described in section 1.a.i. except that STR amplification is not repeated. The information regarding problems with amplification will be noted in the Central lab database (BioLab) since this could represent a highly degraded sample.
2. Indeterminate
   a. Specimen is negative for one marker.
      i. Sample passed QPCR: Repeat amplification one time; if still unsuccessful for one primer set, the family will be asked to submit additional samples, using a Center-approved standard letter. Each family will be asked for no more than one recollect following the standards stated previously. 11 aliquots of the original sample will be made. One large volume aliquot will be subaliquotted into 10 small volume aliquots. The remaining large volume aliquot and the 10 subaliquots will be submitted to CASPIR.
      ii. Sample was considered indeterminate or failed QPCR. Take the same actions described in 2.a.i. except that STR amplification will not be repeated. The information regarding problems with amplification will be noted in the Central lab database (BioLab) since this could represent a highly degraded sample.
   b. Specimen is positive for both markers.

i. Sample was considered indeterminate or failed QPCR. Take same actions described in 2aii.

3. Pass
   a. Specimen is positive for both markers.
      i. Sample passed QPCR. 11 aliquots of the sample will be made. One large volume aliquot will be subaliquotted into 10 small volume aliquots. The remaining large volume aliquot and the 10 subaliquots will be submitted to CASPIR.

Examination of familial relationships to identify possible sample mix-ups and non-paternity / non-maternity will be part of the QC process using the highly heterozygous microsatellite primer sets. Due to low recollect rates and high rates of continued inconsistent results, the Biologics Committee voted in January 2005 to discontinue asking for recollects from families in which the inheritance of the markers was inconsistent with the family relationship. Samples from families determined to have inconsistent family relationships are currently being stored at the Central laboratory.

When samples that received a specimen score of fail or indeterminate following QC analysis are recollected, one set of aliquots will be returned to the originating Center. If aliquots have already been dispersed to Centers from one of the sets, that set of aliquots will remain at CASPIR to maintain consistency across all Centers. If no aliquots have been dispersed, the results of the original samples will be compared to the results of the recollected samples. The samples with better results will remain at CASPIR; the other samples will be removed and returned to the originating Center. This will decrease confusion by maintaining only one set of aliquots at CASPIR for each participant.

Some participants have been identified that have aliquots of DNA collected using both BioTrak and BioKit. The Biologics Committee has determined that the set of aliquots collected using BioKit will remain at CASPIR as long as the specimen score was pass or indeterminate following QC analysis. If the specimen score was fail following QC analysis, the aliquots collected using BioTrak will remain at CASPIR. However, if any aliquots have already been dispersed to Centers from one of the sets, that set of aliquots will remain at CASPIR regardless of the method of collection in order to maintain consistency across all Centers. The set of aliquots that does not remain at CASPIR will be removed and returned to the originating Center.

The Central lab will keep a list of the samples that need to be removed. Once every quarter, the samples will be removed from CASPIR and returned to the Central lab for temporary storage. Once every year the Central lab will return the samples that were removed from CASPIR to the originating Center. The Center may use returned samples in local studies as long as the study complies with the NBDPS informed consent. The Center will store the samples that failed QC in hopes that future technological advances will make them usable.

VIII. Submitting Specimens to CASPIR

All tubes will be labeled using standard NBDPS labels containing the study name, extended family study ID, specimen ID and CDC unique ID (ASTRO). This information links the CASPIR label with the specific Centers study ID (including whether this sample was from an infant, mother or father). For those samples having 11 aliquots at CASPIR, 1 large volume and 10 small volume aliquots, the large volume aliquot has the additional designation "==>50ul<==" on the tube. This aliquot is not available for distribution to Centers but will be stored long-term for future studies. This information will be entered into the Central Lab Database (BioLab) prior to the time the samples are sent by courier to CASPIR. Specimens will be stored at -20°C (or colder) until they are shipped to the CASPIR facility. An electronic file containing specimen information will be sent to the CASPIR facility along with the specimens.

The CASPIR facility will only release specimens on the authorization of the CDC collection custodian (Peggy Honein or Mary Jenkins). This authorization will be given when approvals are obtained as delineated in section IX below.

Some Centers may collect other types of samples (DNA from blood or placenta) on some Centers cases. These samples may be stored at the CASPIR facility and made available for Centers-wide studies if the Center desires. Some Centers may not have storage facilities and may choose to have their samples for local studies stored at the CDC facility. These samples will be made available to the Centers to which they belong without going through the approval process (detailed in section IX below) for Centers-wide studies.

IX. Use of Specimens for Centers-wide Studies

An investigator wishing to use stored samples for a Centers-wide study will follow the procedures defined in the Analysis and Publication Guidelines for the NBDPS Conducted by the Centers for Birth Defects Research and Prevention (CBDRP), previously approved for the Centers.

To initiate a research project, a letter of intent (LOI) must be submitted to the Data Sharing Committee (DSC) to communicate research ideas and facilitate collaboration among Centers. The LOI should follow the guidelines that are delineated in the Analysis and Publication Guidelines. LOIs are reviewed by the DSC. Reviewer comments are compiled and discussed on a DSC conference call. The DSC votes on the LOI and then responds to investigators with their decision. The decision regarding the LOI is also recorded in the Data Sharing database.

Following approval of an LOI, a proposal summarizing the project will be provided to the DSC following the guidelines delineated in the Analysis and Publication Guidelines. Proposals that include use of biologic material must meet further required guidelines located in the same document. The general plan for the project including data sharing and linked analyses will be submitted initially to the Genetic Analysis Working Group (GAWG). The proposals will be reviewed by a minimum of two people from the GAWG. They will be distributed to the GAWG for input but the technical review will come from a minimum of two members. The primary research team can submit the proposals to the GAWG early (a minimum of 2 weeks before the DSC deadline) so the technical review can be attached to it for the DSC to review. If they wait to submit it until the DSC deadline, it will be submitted to the GAWG for review and will not be reviewed by the DSC until the following month. All proposals that have not followed the guidelines will be returned to the submitting lead investigator with a brief note outlining criteria from the Analysis and Publication Guidelines that have not been addressed. The investigator will then be asked to resubmit the proposal with the next data sharing round. For proposals approved prior to Sept 2003, the same review will occur when samples are requested from CASPIR. If the approved proposal does not address the criteria in the Analysis and Publication Guidelines, it will be necessary to submit an updated proposal that addresses these issues.

In addition to the proposal, the ***gene one-pager form*** for the CDC IRB must be completed for each gene to be studied. The genes from one candidate gene pathway may be included on one form if they are not clinically significant. Those genes that are clinically significant must be submitted as separate gene one pagers. The gene one pager forms must accompany the proposal, and approval from the CDC IRB must be obtained before the samples will be released. The CDC IRB review will be expedited and is expected to take 1-2 weeks.

After approval of the research proposal by the DSC, investigators may request biologic samples from CASPIR. Only those samples that are included in the most recent version of the analytic database may be requested. A ***NBDPS Request for Samples from CASPIR*** form must be completed and submitted along with the list of IDs being requested. DNA samples submitted to CASPIR by individual Centers prior to the introduction of the Central lab in Sept 2003 and more recently by the Central lab after May 30, 2005 will be sent directly to the requesting Center. DNA samples processed by the Central lab between Sept 2003 and May 30, 2005 will be returned to the Central Lab and an aliquot will be prepared and sent to the requesting Center.

Limits will be placed on the amount of sample available to any one Center. ***Not more than 10% of the sample sent to the CDC storage facility will be sent to any one Center.*** If a Center desires more of a sample than this, they may collaborate with another Center to obtain additional

samples. At least 20% of the samples stored will be saved for studies far in the future. If a Center requests a sample, an entire tube will be sent. However, if the Center chooses to perform additional studies on this sample, not included in the original proposal, an additional proposal (and gene form for CDC IRB approval) must be sent and approved prior to using the DNA sample for this purpose.

Publication of results from the use of these samples (abstracts and manuscripts) will proceed through the same process established by the Data Sharing committee.

X. External Qualtiy Assessment (EQA)/Proficiency Testing (PT)

The Central Lab staff and any labs that are designated genotyping labs for the NBDPS will undergo periodic EQA/PT according to criteria established in the NBDPS Genotyping EQA/PT Protocol. Specifics of the proficiency testing, and criteria for passing will be established. If the Central lab or designated genotyping lab fails proficiency testing, all extraction and testing will halt until problem areas are identified and remedied. A second round of proficiency testing will be implemented.